Transfer Functions of the Output Promoters of Inducible Systems

Overnight Incubation

Complete for each strain in a biohood. Throughout the procedure it is important to label all materials so that the strains do not get confused.

1. Pipette 4 mL sterile LB/antibiotic media to a 14 mL culture tube for each strain. Also pipette 4 mL sterile LB media to a 14 mL culture tube for DH10B.
    a. Pgk – 4mL LB + 4 µL kan
    b. Pck – 4mL LB + 4 µL kan
2. Using a sterile tip transfer frozen stock to the corresponding culture tube.
3. Place the cap on the culture tubes to the first position to allow aeration.
4. Place the tubes into the incubator and allow them to grow overnight (~ 16 hours) at 37°C and 250 rpm.

Dilution

5. Measure OD at 600 nm in cuvettes for each culture ($OD_{O/N}$) (900 µL water and 100 µL culture well mixed) $OD_{O/N}$ = absorbance from Tecan * 10
6. Dilute the cultures to OD of 0.1 in a total of 4 mL fresh LB/antibiotic media (just LB for the DH10B) in new 14mL culture tubes.
    a. Pck and pgk both use kan
    b. Make a 13mL dilution using the equation below for each culture (3 total)

    Volume of overnight culture ($V_{O/N}$) = 0.1*13mL/$OD_{O/N}$

    Volume of LB/ antibiotic (just LB for the DH10B) = 13mL – $V_{O/N}$
7. Incubate 1.5 - 2 hours at 37°C and 250 rpm.
8. Measure OD at 600 nm in cuvettes for each culture (OD_{DIL}) and record.
    - Prepare LB/antibiotic mixture
        - Will need **120mL LB + 120 mL kan**

Induction (can do steps 9 - 15 while waiting for step 7)

9. For each inducer, label 3 50mL conicals with the 125x serial dilutions of the maximum inducer concentration, Table 1.

		Tube 9 (4)	~~Tube 8~~	~~Tube 7~~	**Tube 6 (3)**	~~Tube 5~~	~~Tube 4~~	**Tube 3 (2)**	~~Tube 2~~
Inducer (I)	Stock [I]	**Max [I]**	~~5x [I]~~	~~25x [I]~~	**125x [I]**	~~625x [I]~~	~~3125x [I]~~	**15625 x [I]**	~~78125 x [I]~~
Ara	1 M	**25 mM**	~~5 mM~~	~~1 mM~~	**200 µM**	~~40 µM~~	~~8 µM~~	**1.6 µM**	~~320 nM~~

Table 1 Diluted Inducer Concentrations

Arabinose (Ara),

10. Calculate volume to take from the inducer stock to achieve the max [I] for each inducer with the following equation.

    V_{stock} = (**30L** total in tube) * (25mM [Ara]) * (4mL total in culture growth tubes)/((1000mM [Ara])*(3.33 mL inducer/LB/ antibiotic mix in tubes)

    = 0.9009mL Ara

Inducer	Ara (original)	Ara (using new equation above)
V_{stock}	30 µL	900.9 µL 1M Ara

Table 2 Inducer Stock and LB/ antibiotic Volume Per Tube 9

11. Add V_{stock} and 29.1 mL LB/antibiotic to tube 4. Vortex to mix well.
12. Add 29.76 mL LB/antibiotic to tubes 2 and 3.
13. Remove 240 uL from each tube 4 and add to tube 3, then vortex. Repeat, 240 uL from tube 3 to tube 2, vortexing each time.
14. In 24 tubes, add 3.33 mL from each tube to its corresponding tube, see Table at the end. In 3 tubes (tube 1 of each set) add 3.33 mL LB/antibiotic with no inducer.

pBAD/ Ara

Position	Tube 1	Tube 2	Tube 3	Tube 4
Tube and [I]	LB/antibiotic 0 inducer	1.6 µM Ara	200 µM Ara	Max [Ara]

Table 3 Inducer Concentration Per Tube

15. Add 3.33 mL LB to Tubes G1, G2 and G3
16. Add 0.67mL cell culture to the tubes, see Table 3. Add 0.67 mL DH10B to Tubes G1 – G3.
17. Put tubes on shaker
18. Incubate 7-8 hours at 37°C and 250 rpm.
19. Conduct appropriate assay.

		1	2	3	4
Pck	A	No inducer	Tube 2	Tube 3	Tube 4
Pck	B	No inducer	Tube 2	Tube 3	Tube 4
Pck	C	No inducer	Tube 2	Tube 3	Tube 4
pgk	D	No inducer	Tube 2	Tube 3	Tube 4
pgk	E	No inducer	Tube 2	Tube 3	Tube 4
pgk	F	No inducer	Tube 2	Tube 3	Tube 4
DH10B	G	No inducer	No inducer	No inducer	
	H				

Extraction

Tris extraction protocol:

1. Prepare extraction solution of 0.1 M Tris and 2 mM EDTA. Adjust to pH 7.75 with acetic acid. For 27 samples, we will need about 26 mL.
2. Pellet the induction cultures by centrifuging at 3000g for 12 minutes.
3. Pour off the supernatant and resuspend each culture in 100 µL of PBS.
4. For each sample, aliquot 900 µL of Tris/EDTA solution into an eppendorf tube.
5. Place the eppendorf tubes into floaters.
6. Heat the Tris/EDTA tubes in a boiling water bath, set for two minutes at boiling
7. Add the 100 µL of each sample to the Tris/EDTA tubes.
8. Let sit in the boiling water bath for 1 minute.
9. Remove tubes from boiling water bath and allow to cool to room temperature. Store on ice or in a -20C freezer for longer periods.
10. Pellet the debris by centrifuging at 16000g for 5 minutes.
11. Transfer supernatant to new ep tubes. Store what you do not use for the assay at -20°C.

Reagent Preparation (Wednesday)

1. Make 1.0 mL of *1X Reaction Buffer*
    - Fill 1.5 mL tube with 950 µL dH_2O
    - Mix in 50 µL of *20X Reaction Buffer* (Component E)
2. Make 1 mL of a 10 mM *D-luciferin stock solution*
    - Use 1 mL of the *1X Reaction Buffer* (all of it)
    - Add to a vial of *D-luciferin* (Component A, blue cap)
    - Protect from light until use
    - Lasts for a couple of weeks at ≤–20°C away from light
3. Prepare a 100 mM *DTT stock solution*
    - Use bottle containing 25 mg of *DTT* (Component C, black cap).
    - Add 1.62 mL of dH_2O to the bottle
    - Aliquot into ten 160 µL volumes and store frozen at ≤–20°C
    - To use, thaw in the 4°C and keep on ice
4. Prepare low-concentration *ATP standard solutions* (range is 1nM to 1uM ATP)
    - Dilute the 5 mM *ATP solution* (Component D, green cap) in dH_2O
        i. Add 1µL into 5 mL H20 (1000nM ATP)
        ii. Take 500 µL from 1000nM ATP and add to 4.5mL H20 (100nM ATP)
        iii. Repeat 2 times (making 10nM and 1nM ATP
    - Solutions are stable for several weeks when stored at ≤–20°C.

Standard Reaction Solution (Late Thursday)

1. Thaw necessary reagents in 4°C
    - A single DDT aliquot (160 µL)
    - 4 ATP Standards
2. Make 10 mL of a *Standard Reaction Solution* (we can alter this formula)
    - 8.9 mL dH_2O
        - 4450 µL from purple pipette twice
    - 0.5 mL *20X Reaction Buffer* (Component E)
    - 0.1 mL *0.1 M DTT* (Reagent Step 3)
    - 0.5 mL of *10 mM D-luciferin* (Reagent Step 2)
        - store the remaining 0.5 mL at ≤–20°C for up to several weeks
    - 2.5 µL of *Firefly Luciferase 5 mg/mL stock solution*
3. Gently invert the tube to mix
    - Do not vortex
    - Keep the reaction solution protected from light until use.
    - Although the solution may be stored at 2–6°C protected from light for several days, assay sensitivity will diminish with time.

	1	2	3	4	5	6	7
A	20µL lysate 180µL SRS	20µL lysate 180µL SRS	20µL lysate 180µL SRS	20µL lysate 180µL SRS	No sample 200µL SRS	No sample 200µL SRS	No sample 200µL SRS
B	20µL lysate 180µL SRS	20µL lysate 180µL SRS	20µL lysate 180µL SRS	20µL lysate 180µL SRS	20µL 1nm ATP 180µL SRS	20µL 1nm ATP 180µL SRS	20µL 1nm ATP 180µL SRS
C	20µL lysate 180µL SRS	20µL lysate 180µL SRS	20µL lysate 180µL SRS	20µL lysate 180µL SRS	20µL 10nm ATP 180µL SRS	20µL 10nm ATP 180µL SRS	20µL 10nm ATP 180µL SRS
D	20µL lysate 180µL SRS	20µL lysate 180µL SRS	20µL lysate 180µL SRS	20µL lysate 180µL SRS	20µL 100nm ATP 180µL SRS	20µL 100nm ATP 180µL SRS	20µL 100nm ATP 180µL SRS
E	20µL lysate 180µL SRS	20µL lysate 180µL SRS	20µL lysate 180µL SRS	20µL lysate 180µL SRS	20µL 1000nm ATP	20µL 1000nm ATP	20µL 1000nm ATP

					180μL SRS	180μL SRS	180μL SRS
F	20μL lysate 180μL SRS	20μL lysate 180μL SRS	20μL lysate 180μL SRS	20μL lysate 180μL SRS			
G	20μL lysate 180μL SRS	20μL lysate 180μL SRS	20μL lysate 180μL SRS				
H							

Standard Curve

1. Place 200μL of the *Standard Reaction Solution* in the luminometer and measure the background luminescence.
2. For each sample, start the reaction by adding 20μL of *dilute ATP standard solution* to 180μL of *Standard Reaction Solution* and read the luminescence.
    - The volume of the dilute ATP standard solution that is added to the standard assay solution should be no more than 10% of the total assay volume
3. Subtract the background luminescence.
4. Generate a standard curve for a series of ATP concentrations.
    - Be sure to always add a constant sample volume of the ATP containing solution

Sample Analysis

1. Follow the directions given in Standard Curve, substituting *ATP-containing samples* for the *ATP standard solutions*.
    - The total volume of the experimental sample assays should be equal to that of the ATP standard assays
    - The amount of sample added should amount to no more than 10% of the total assay volume.
2. Calculate the amount of ATP in the experimental samples from the standard curve.